Journal of Immunological Methods, 76 (1985) 365–373
Elsevier

P.D. 1985
P. 365-373 (9)

JIM 03376

XP-002150865

Direct Measurement of Antibody Production in Cell Suspensions Using an Enzyme-Linked Immunosorbent Assay

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(Received 14 September 1984, accepted 18 October 1984)

We have developed an enzyme-linked immunosorbent assay (ELISA) to measure antibody production in cell suspensions using highly sensitive avidin-biotinylated peroxidase (ABC) reagents. The cells are serially diluted in media and placed directly on a standard antigen-coated microtiter plate for 6 h, and the plate is then washed and processed to determine the bound antibody. The amount of antibody detected is reduced by puromycin indicating in vitro synthesis. Standard errors of the means of optical density readings of replicate samples are less than 10%. The background readings and readings obtained using non-immune cells are negligible, demonstrating no significant contamination by endogenous peroxidases.

Key words: *ELISA – cells – antibody production – avidin-biotin – antibody synthesis*

Introduction

Since its introduction by Jerne and Nordin (1963), the hemolytic plaque assay has become a widely accepted method of estimating antibody production in cell populations. Its low cost and simplicity are frequently offset, however, by a troublesome degree of variability, due primarily to the requirement for the use of biological reagents in the hemolytic process. In addition, the procedure of counting individual plaques is inaccurate and time-consuming. Recently 2 laboratories have reported an ELISA analog to the plaque assay in which immunochemical reagents are substituted for the more variable biological elements (Czerkinsky et al., 1983; Sedgwick and Holt, 1983). While this method eliminates the use of complement and target cells, there nevertheless remains the necessity of laborious tabulation of data. We have developed an ELISA in which the amount of antibody being produced in a cell population can be quickly and reproducibly determined. Antibody production by different cell populations can then be compared relative to a standardized positive

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control antiserum included on each plate. While this method does not give data in the form of numbers of antibody-producing cells, the overall synthetic capacity of a cell population may have equal biological relevance.

Materials and Methods

Antigen and immunization procedures

Fowl gamma globulin (FGG) was purchased from Cappel Laboratories (Cooper Biomedical, cat. no. 0004-0910). This material was determined by gradient polyacrylamide gel electrophoresis to be greater than 90% pure chicken IgG with minor contamination by a high molecular weight species probably corresponding to 19 S chicken immunoglobulin.

Female ICR mice purchased from Harlan Sprague-Dawley were used in cell experiments at 8–20 weeks of age. Mice were immunized by intraperitoneal (i.p.) injection of 0.2 ml saline containing 100 μg FGG and 10^9 formalin-fixed *Bordetella pertussis* organisms. Where anamnestic responses were to be measured, the animals were boosted i.p. with the same mixture 1 month after primary stimulation.

Cells

Mice were sacrificed 1–2 weeks after primary or secondary immunization and spleens were excised and minced under sterile conditions. The cells were suspended in 10 ml Hanks' balanced salt solution and filtered through a sterile 110 μm nylon mesh filter (Nitex, Tetco, Elmsford, NY). Cells were washed by centrifugation for 10 min at $400 \times g$ and resuspended in 10 ml RPMI 1640 (Gibco) at 4°C containing 10% heat-inactivated fetal calf serum, 150 U/ml penicillin and 150 μg/ml streptomycin. The same medium containing 1–5 μg/ml puromycin (Sigma) was used in parts of some experiments. Where purification of nucleated cells was done, red cells were removed by layering 7 ml of the cell suspension over a Ficoll-Hypaque gradient (Histopaque 1077, Sigma) and centrifuging at $400 \times g$ for 30 min. Elimination of antibody carryover was accomplished either by multiple washing of cells in media or by centrifugation twice through a 100% fetal calf serum gradient. The latter procedure was accomplished by layering 7 ml of cells over 3 ml fetal calf serum and centrifuging at $400 \times g$ for 10 min. Lysates of cell suspensions were prepared by freeze-thawing aliquots of cells in liquid nitrogen, sonicating briefly in an ultrasound waterbath (Bransonic 32, Bransonic Cleaning Equipment Co., Shelton, CN) and then clearing the resulting suspension by centrifugation.

ELISA for plasma antibody against FGG

Ninety-six-well microtiter plates (Linbro) were coated overnight with 1 μg/ml FGG in carbonate buffer (0.015 M Na_2CO_3, 0.035 M $NaHPO_4$, pH 9.6). Plates were then washed 5 times with phosphate-buffered saline (Gibco) containing 0.05% Tween 20 (PBS/Tween) using an automated microtiter plate Multiwash (Flow Laboratories). Aliquots of dilutions of FGG-immune plasma made up in PBS/Tween were then added to the top row and serially diluted in the lower rows. Plates were

incubated overnight at 4°C. Plates were then washed and 100 μl of biotinylated horse anti-mouse IgG (Vector Laboratories) were added to each well. Following a 2 h incubation at 4°C, plates were washed and 100 μl of a solution containing avidin-biotinylated peroxidase complexes (ABC) (Vector Laboratories) were added to all wells. After 30 min at room temperature plates were washed and colored with orthophenylenediamine in a vibrating room temperature water bath. The coloration buffer is a citrate buffer (0.05 M citric acid, 0.1 M Na_2HPO_4, pH 5.0) to 100 ml of which is added 40 mg *o*-phenylenediamine (Sigma) and 40 μl of 30% H_2O_2. The reaction was stopped after 10 min with 100 μl 2.5 M H_2SO_4 and optical densities were read at 492 nm using a Titertek Multiskan microtiter plate photometer (Flow Laboratories). Standard errors of the means of triplicate readings are less than 10% of the mean values. We store and analyze ELISA data with the aid of a Commodore 64 microcomputer (Commodore Business Machines) interfaced to the Titertek Multiskan. By means of a program which determines the dilution at any desired optical density endpoint assuming linearity between the 2 points bracketing the endpoint, the dilution of the standard antiserum and cells giving the specified optical density can be determined (DiRusso et al., 1985).

Standard antiserum

Pooled heparinized plasma obtained from mice 4–8 weeks post-immunization was heat-inactivated and filtered through a 0.2 μm filter. The antiserum produced a titer of approximately 20,000 on FGG-coated microtiter wells and essentially background readings on wells coated with an irrelevant antigen (bovine serum albumin). The titer could be reduced by more than 90% by prior incubation with 100 μg/ml soluble FGG before addition to the plate. The amount of specific antibody was determined to be approximately 40 μg/ml by radial immunodiffusion in agar against known concentrations of FGG in 8 replicates. Concentrations of protein were determined by a Coomassie protein assay (Bio-Rad). Equal volumes of antiserum and antigen solutions were added to die-cut wells in 0.6% agarose (Sea-Kem) containing 0.02% sodium azide. The plates were incubated for 48 h at room temperature in a humidified chamber. The gels were then allowed to stand overnight in 10% NaCl, washed thoroughly with deionized water and stained with Coomassie blue. The distance of the precipitin line from the central well containing antiserum was measured twice for each of 8 replicates using an eyepiece micrometer. Since the molecular weight of chicken IgG is 170,000 (Leslie and Clem, 1969), formation of a precipitin line at 50% of the distance between the 2 wells was taken to represent equal concentrations of antibody and antigen (Williams, 1971).

Modification of ELISA for cells

Cells were maintained at 4°C before addition to plates, and addition was performed in a 4°C cold room. Prior to the addition of cells or supernatants from cell washes, plates were washed with PBS/Tween. The use of this buffer to wash the plates was not toxic to the cells since the antibody produced did not decrease from that observed if Tween was omitted. Two hundred microliters of cells at 1–2 × 10^7 nucleated cells/ml or lysates of the same suspensions were added in triplicate to the

top row of the plate. One hundred microliters of the appropriate medium were added to the remaining wells and serial 2-fold dilutions were performed on the plate using a 12-channel pipet. Alternatively, dilutions were performed in a single tube and cells were then transferred to each row after each dilution. We found that the latter procedure eliminated a problem with disproportionately high rates of decrease in the optical density readings which were apparently due to adherence of antibody-producing cells to the antigen-coated wells during serial dilution on the plate. The standard antiserum was titered in media in replicates on each plate and used to quantitate antibody production by cells incubated on the same plate. The plates were incubated for 6 h at 37°C in a humidified CO_2 incubator. They were then washed and 100 μl of the biotinylated second antibody were added to each well. The remaining steps were exactly as described for plasma antibody.

Results

Assay for in vitro antibody production

We found that antibody production by immune spleen and lymph node cell suspensions was readily measured by an ABC ELISA (Fig. 1). One problem we experienced was the carryover of plasma antibody in spleen cell suspensions (Fig. 2). Elimination of significant amounts of antibody in the supernatant from the last wash required 5 or more washes in the case of anamnestically stimulated spleen cells. In testing different methods of eliminating carryover, we found that centrifugation through 2 fetal calf serum gradients eliminated > 95% of the antibody in the final supernatant provided all steps were carried out at 4°C. In 1 experiment using 5 animals, the average increase in optical density readings due to antibody in the final supernatant was 0.124 (range 0.027–0.218) over a background reading of 0.073.

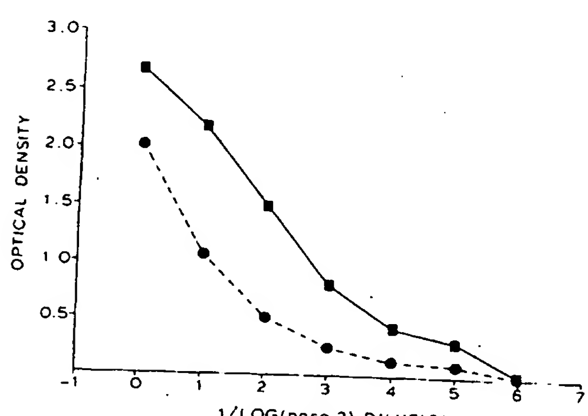

Fig. 1. Antibody production by cells in vitro. Anamnestically stimulated spleen cells were washed 5 times (■) and then titrated on the plate with 10^6 cells in the top row. The supernatant from the last wash ( ) was also diluted in replicates. All optical density readings represent the mean of 3 replicates.

Plasma antibody dilutions from the same animals yielding an optical density of 0.124 were in the range of 50,000–150,000. Therefore, assuming the initial cell suspension to be a 1 : 500 dilution of plasma antibody, less than 1% of the initial antibody remained in the final supernatant.

A second problem was the in vitro secretion of antibody by the cells during the lengthy process of preparing them for the antibody assay. The cells should be equilibrated at 4°C after initial suspension in order to decrease the rate of antibody synthesis and secretion. At first we suspected that contamination of the plate by cells containing endogenous peroxidases might prove to be a problem, but we found that non-immune cells gave essentially background readings. In addition, no cells could be observed remaining on the plate by microscopic examination prior to the addition of enzyme substrate.

Inhibition of antibody synthesis by puromycin

The antibiotic puromycin, which causes premature polypeptide chain termination, was used to determine what proportion of the antibody being measured was synthesized in vitro. Incubation in media containing 1 μg/ml puromycin during the 6 h at 37°C produced a decrease in optical densities observed and preincubation with puromycin for 1 h at 4°C produced a further drop (Fig. 3). The viability of cells as determined by trypan blue exclusion was the same among all 3 groups after the incubation period, and puromycin did not affect the binding of antibody when added to the control antiserum. An alternative explanation for this observation is that the inhibition seen was due to inhibition of a protein required for the transport of intracellular immunoglobulin. We ruled out this possibility through the use of cell lysates before and after incubation with and without puromycin (Fig. 4).

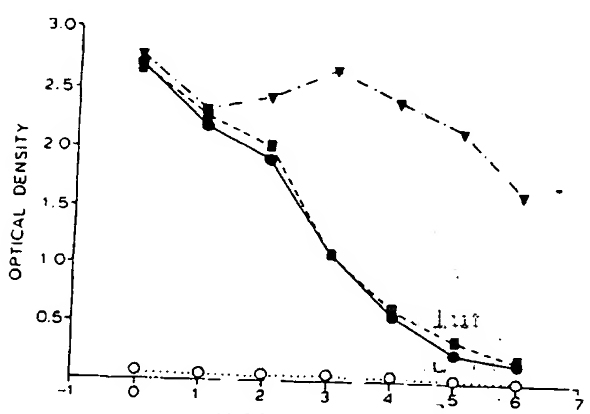

Fig. 2. Antibody carryover in cell suspensions. Significant antibody carryover can be observed even after cells are washed 3 times. Spleen cells from an anamnestically stimulated (●) and a naive mouse (○) were washed 3 times and serially diluted on an antigen-coated microtiter plate with 10^6 cells in the top row. The supernatant from the third wash of the immune cells was added either by itself (▼) or with non-immune cells (■). Each optical density value is the mean of 3 replicates.

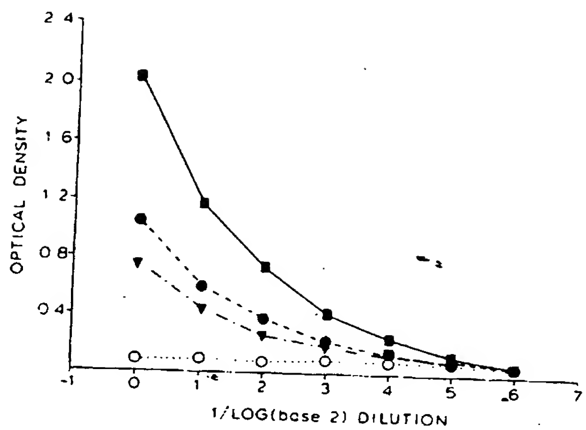

Fig. 3. Effect of puromycin. Spleen cells isolated from a mouse 14 days following primary stimulation were purified over a Ficoll gradient, divided into 3 portions, and washed a total of 7 times in RPMI. Cells were serially diluted in media on the microtiter plate with the top row containing 10^6 cells. One portion (▼) was preincubated for 30 min in medium containing 1 μg/ml puromycin prior to the seventh wash. Aliquots from the first portion and a second portion (●) were incubated on the microtiter plate in medium containing puromycin. The third portion (■) was incubated in medium only. The supernatant from the seventh wash of the untreated cells (○) was also added to the plate.

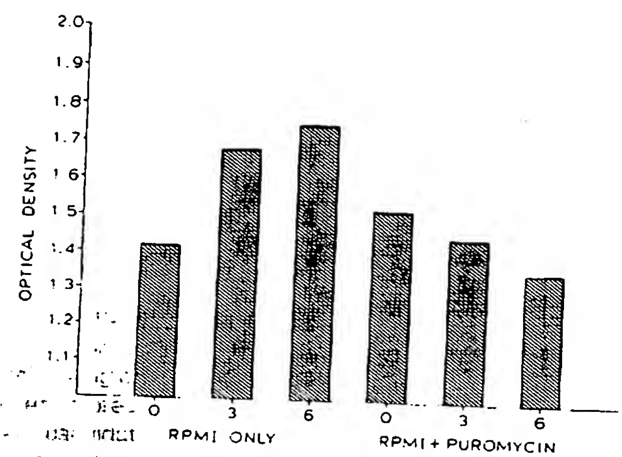

Fig. 4. Timecourse of antibody production. Spleen cells were obtained from a mouse 1 week after secondary immunization. Lysates were prepared from cells at 10^7/ml incubated at 37°C either in the presence or absence of 5 μg/ml puromycin. Aliquots of cells were taken and the lysates assayed for antibody content at times 0, 3, and 6 h. After 6 h the viability of cells in medium or medium containing puromycin was found to be almost identical (~80%) by trypan blue exclusion. Each optical density reading represents the mean of 2 replicates at the equivalent of 5×10^5 nucleated cells/well.

Quantitation of antibody production in vitro

The standard control antiserum was used to quantitate antibody production in cell populations (Fig. 5). Since the amount of precipitable antibody in the standard is known and equal amounts of bound antibody will give equal optical density readings, the antibody produced by a given number of cells can be calculated. Bound antibody in the subnanogram range can be quantitated using this procedure.

Discussion

The use of ABC reagents to increase the sensitivity of ELISA permits the direct assay of the rate of immunoglobulin secretion by immune spleen cells even at very low levels of antibody secretion. The reagents used have a low background, and non-immune cells do not contribute to readings. Among the advantages of this assay system are speed due to automation, reproducibility due to the use of standard immunochemical reagents, and sensitivity due to ABC amplification. Using the titers obtained with the standard antiserum, the assay proved to be highly reproducible when performed under standard conditions. Between run coefficients of variation average about 10%. Within a given assay the standard error of the means of replicate values is less than 10% of the mean. The high degree of sensitivity permits small numbers of cells, for instance $1-2 \times 10^6$ immune spleen cells or lymph node cells, to be assayed for antibody production. The antibody titers obtained compared favora-

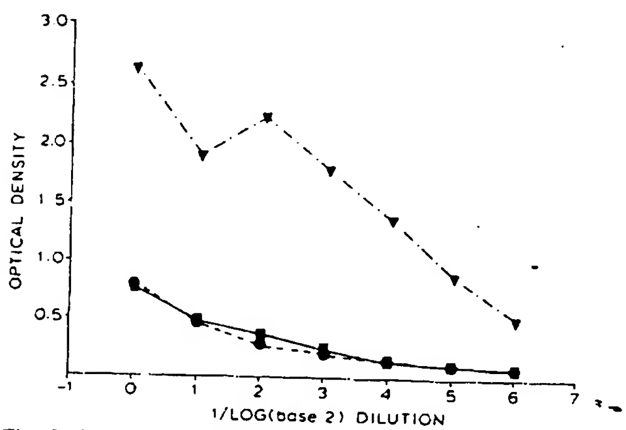

Fig. 5. Quantitation of antibody production in vitro. Spleen cells isolated from 2 mice 20 days after primary immunization were centrifuged twice through a fetal calf serum gradient and diluted in replicates on an antigen-coated plate with 2×10^6 cells in the top well (■,). A 1:500 dilution of control antiserum was also added to the plate (▼). The number of nucleated cells yielding an optical density reading of 0.6 was calculated to be 1.3×10^6 for both animals. The corresponding amount of precipitable antibody in the antiserum was determined to be 140 pg. Therefore the amount of antibody produced in the 2 samples is approximately 100 pg/10^6 nucleated cells.

bly in relative magnitude with those obtained from concentrated culture supernatants from 5 day cell cultures.

Under less than optimal culture conditions, antibody production may shut down within hours after cells are placed in culture. Czerkinsky et al. (1983) found that with high numbers of cells (10^6), antibody detected in cell cultures seems to plateau and even decrease after 4 h. This is also in agreement with observations by Van Furth (1966). Time course experiments using this assay system suggest that most antibody is secreted within the first 3 h of culture (Fig. 4). This phenomenon could be due to the presence of a low frequency inhibitory cell population or perhaps to the secretion of an inhibitory factor by B cells themselves as recently described by Kiely et al. (1984). It is very likely, however, that the apparent shutdown of immunoglobulin production is due to suboptimal culture conditions. If so then this technique should prove useful in optimizing culture conditions to provide for sustained synthesis.

Studies using plasmacytoma cell lines have yielded estimates that a single antibody-producing cell synthesizes 1–10 pg antibody during a 6 h period (Askonas, 1961; Humphrey and Fahey, 1961; Nossal and Mäkelä, 1962). In the example illustrated in Fig. 5, the spleen of 1 animal yielded 90×10^6 and that of the other yielded 100×10^6 viable nucleated cells. Therefore, since antibody production was seen to be approximately 100 pg/6 h for both animals, the total number of antibody-secreting cells/spleen can be estimated to be in the range of 10^3–10^4. This is a low but not unreasonable figure for the primary murine IgG response to FGG at 20 days (Miller and Warner, 1971).

It is interesting to note the relatively high amount of antibody which can be carried over in supernatants even after 3 cell washes. As can be seen from Fig. 2, this amount can exceed by several 2-fold dilutions that are produced by the cells. The plasma antibody from the immunized animal illustrated in Fig. 2 produced a titer of over one million using the ABC reagents. If one considers the hemolytic plaque assay, where cells washes are usually carried out only to free the suspension of contaminating red cells (Jerne et al., 1974), one must come to the conclusion that frequently the amount of plasma antibody far exceeds that produced by the cells when they are plated out. This, then, constitutes yet another contribution to the variability which may be encountered when employing the plaque assay.

We acknowledge that the data yielded by this assay differ in a basic way from that produced in plaque assays. Using this assay one can arrive at numbers of antibody-forming cells per unit of cells only in an indirect way. This technique may be useful, however, in those instances where the investigator is primarily interested in the relative antibody-producing capacities of different cell suspensions and not specifically how many cells are involved. An additional, interesting application is suggested by the apparent absorption of plasma antibody by the non-immune cells shown in Fig. 2. Measurement of Fc receptor-mediated uptake and non-specific degradation might be conveniently performed using competitive and non-competitive inhibitors. The sensitivity of the ELISA may not extend to the low range of that enjoyed by the plaque assay. However, if the level of immunization is sufficient, we believe that this method offers an attractive alternative to the hemolytic plaque assay.

Conclusion

Antibody produced by cells during incubation on an antigen-coated microtiter plate can be measured by ELISA using avidin-biotinylated peroxidase complex reagents. The reproducibility of the technique is excellent as shown by the low variability seen within triplicate wells. The assay has low background readings, approximately 0.06 on the Titertek Multiskan. Despite the high degree of sensitivity there is no problem with non-specific contamination by intracellular peroxidases as evidenced by the low readings obtained using unfractionated naive spleen cells. That antibody detected is due largely to release of in vitro synthesized antibody is shown by inhibition of detectable antibody by puromycin, a specific inhibitor of protein synthesis.

Acknowledgements

We extend our thanks to Dr. Robert Jerris for assistance in preparing a pertussis vaccine for use in immunization and to Dr. Irene Check for helpful suggestions and discussion.

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